Flagging Drugs That Inhibit the Bile Salt Export Pump

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Article

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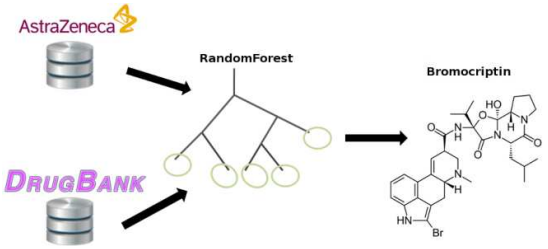

Abstract

The bile salt export pump (BSEP) is an ABC-transporter expressed at the canalicular membrane of hepatocytes. Its physiological role is to expel bile salts into the canaliculi from where they drain into the bile duct. Inhibition of this transporter may lead to intrahepatic cholestasis. Predictive computational models of BSEP inhibition may allow for fast identification of potentially harmful compounds in large databases.

This article presents a predictive *in silico* model based on physico-chemical descriptors that is able to flag compounds as potential BSEP inhibitors. This model was built using a training set of 670 compounds with available BSEP inhibition potencies. It successfully predicted BSEP inhibition for two independent test sets and was in a further step used for a virtual screening experiment. After *in vitro* testing of selected candidates, a marketed drug, bromocriptin, was identified for the first time as BSEP inhibitor. This demonstrates the usefulness of the model to identify new BSEP inhibitors, and therefore potential cholestasis perpetrators.

Keywords

BSEP, classification model, inhibition, drug-induced cholestasis, bromocriptin

Introduction

The bile salt export pump (BSEP; gene name *ABCB11*) is a member of the subfamily B of ABC transporters. It is expressed in the liverat the canalicular membrane of the hepatocytes. It transports bile salts from the hepatocytes into the bile using the energy provided by ATP hydrolysis. The main substrates of BSEP are monovalent conjugated bile acids such as taurochenodeoxycholate [1]. However, drugs such as pravastatin have also been shown to be transported by BSEP (although with low affinity and therefore without *in vivo* impact) [2].

Impairment of BSEP expression or function due to mutations in the *ABCB11* gene leads to progressive familial intrahepatic cholestasis type 2 (PFIC2) [3]. PFIC2 is an autosomal recessive condition which starts at early childhood with mild cholestasis, but develops in more critical liver failures and may necessitate liver transplantation at later stages.

Inhibition of BSEP by small molecules may also impair its function. As a result, efflux of bile salts decreases, leading to their accumulation inside the hepatocytes and, consequently, to drug-induced liver injury (DILI) manifested as acquired intrahepatic cholestasis. Bosentan [4] and troglitazone [5] are two examples of drugs whose hepatotoxicity can be directly linked to an inhibition of BSEP. The latter was withdrawn from the market in 2000 for its toxicity.

While the Food and Drugs Administration (FDA) does not provide directives regarding the testing of drugs for their inhibitory activity on BSEP, the European Medicine Agency (EMA) suggests investigating potential inhibition of the transporter [6,7]. In case *in vitro* studies reveal an inhibition, the serum bile salt levels should be monitored *in vivo* during later phases in the drug development process. In this scenario, a fast and inexpensive *in silico* pre-screening of candidates could be useful in order to reduce the amount of *in vitro* tests needed.

There are few *in silico* models of BSEP inhibition available so far. The model of Hirano and colleagues [8] is based on a very small data set of 37 compounds and thus would not allow *in silico* profiling of chemically diverse compound libraries. Warner *et al*. [9] used a balanced training set of 437 compounds to build several classification models based on in-house descriptor sets, comprising 2D, 3D and fingerprint-like features. Their best model reached an accuracy of 0.87 (on a test set of 187 compounds). Finally, Pedersen and colleagues [10] built two orthogonal partial least-squares discriminant analysis (OPLS-DA) models on 163 compounds. They report an accuracy of 0.89 on a test set of randomly selected 86 compounds. However, none of these models were used in any prospective studies to identify new BSEP inhibitors and thereby demonstrate their performance in real life settings.

In this study, we built a classification model based on a set of physico-chemical descriptors for predicting BSEP inhibition. The model revealed the importance of hydrophobicity, aromaticity and H-bond donor characteristics for separating inhibitors from non-inhibitors. It had an accuracy of 84% in ten-fold cross-validation, 80% in a randomly selected external set, and 88% in a validation set built on publicly available data. A subsequent screen of the DrugBank database revealed many drugs already known as BSEP inhibitors in the top-ranked positions. To further evaluate the prospective usefulness of the model, we selected compounds from the top-scoring ones for which up to now no BSEP inhibition information was available. Selection criteria comprised, among others: chemical diversity, known bile elimination or liver metabolism, and commercial availability. Finally, seven compounds were experimentally tested using two distinct *in vitro* systems: a membrane vesicle assay and a more complex Transwell® assay. This led us to identify bromocriptin, a currently marketed drug, as BSEP inhibitor. The model has been implemented as a KNIME workflow [12] which is available for download from the supplementary material.

Material and methods

The original data was donated by AstraZeneca within the framework of the IMI project eTOX (www.etoxproject.eu). It contained the BSEP inhibitory potencies reported as IC_{50} (μM) values of 1092 compounds provided as smile strings. Data was measured in a [^{3}H]-taurocholate transport assay performed in Sf21 membrane vesicles following the protocol in [13].

Database curation

The initial dataset was curated using the procedure described in Pinto *et al*. [14]. Briefly, it consists of the following steps: (1) removal of inorganic compounds (*i.e*. compounds not containing carbon atoms) using Instant JChem v.5.3, 2010, ChemAxon (http://www.chemaxon.com); (2) analysis and removal of mixtures formed by two or more large molecules; (3) deletion of organometallic compounds using MOE 2011.10 [15]; (4) identification and removal of compounds containing special atoms such as selenium or tellurium by means of an in-house MOE SVL script; (5) normalization of chemotypes using the ChemAxon's Standardizer with the following settings: clean 2D, aromatize, mesomerize, neutralize, tautomerize and all transform options; (6) identification and elimination of non-unique structures using MOE; (7) deletion of compounds having permanent charges. The application of this procedure led to a curated data set of 1048 compounds.

Compound labeling

Compounds having an IC_{50} lower or equal to 10 µM were considered as inhibitors, whereas those having an IC_{50} higher than 50 µM were labeled as non-inhibitors. Compounds having activity values between 10 and 50 µM were not considered to build the classification models and were discarded. This procedure led to a final data set formed by 838 compounds, 563 of which were non-inhibitors and 275 were inhibitors.

Descriptor calculation

78 interpretable 1D and 2D descriptors representing physico-chemical properties were calculated using MOE2011.10 [15]. These descriptors were not selected based on information content or correlation with the dependent variable, but rather on their physico-chemical interpretability. The exact list of descriptors is available as supplementary information. Descriptors of the training set were Z-normalized using KNIME [12]. Computed means and standard deviations were then applied to the different test sets (see below).

Training and test set selection

80% of each class was randomly selected for use as training set. The remaining compounds were considered as test set. The composition of the resulting sets is shown in Table 1.

	Total	Inhibitors	Non inhibitors
Complete dataset	838	275	563
Training set	670	220	450
Test set	168	55	113
External set	156	39	117

Table 1: Composition of the datasets in number of compounds

External validation

In order to assess the prospective predictive power of the model, an external validation was carried out using a data set compiled from the literature [8,16]. Hirano *et al.* [8] tested 42 compounds among neurotransmitters, calcium channel blockers, potassium channel modulators, steroids, non-steroidal anti-inflammatory drugs, anticancer drugs, and others. The percentage of inhibition of BSEP at 100 µM was measured (in comparison with a negative control without test drug). The highest inhibition was obtained for nicardipine (100% inhibition), prenylamine (77% inhibition), fendiline (80% inhibition) and nifedipine (75% inhibition) from the calcium channel blockers, while the other compounds showed low to moderate inhibition (under 50%). Those were therefore not considered as inhibitors.

Morgan *et al.* [16] measured the BSEP inhibition activity of 217 compounds to relate them with liver injuries. Compounds with IC_{50} below 25 µM were taken as inhibitors, while compounds with IC_{50} over 135 µM were taken as non-inhibitors. The 25 µM threshold was chosen because, among the compounds that were present both in the training set and in [16], the equivalent IC_{50} in the training set was usually below 10 µM.

The chemical structures were retrieved by name from Pubchem Compound (http://www.ncbi.nlm.nih.gov/pccompound). This external set was curated using the same protocol as previously described. Those compounds also present in the AstraZeneca data set

were removed, leading to a final set formed by 156 compounds, 39 inhibitors and 117 non-inhibitors.

Model building

The Weka 3.7.10 library [17] was used to build a set of classification models. The machine learning algorithms Naïve Bayes (NB), k-nearest neighbor (IBk), J48 and Random Forest (RF) were used with embedded attribute selection (CfsSubsetEval evaluator and BestFirst search). Additionally, in order to improve the performance of the models, optimization of the number of neighbors in IBk, the minimum number of instances per leaf in J48 or the number of trees in RF was performed using the CVParameter selection tool provided by Weka with an internal 5-fold cross-validation.

The model showing best performance was implemented as a KNIME workflow [12]. It is available for download in the supplementary information. KNIME is an Open Software platform used for implementing pipeline-like workflows. Here, the pipeline starts by reading the input dataset (as an sd file), then computes the required descriptors using the MOE node. The trained model is then read and the new compounds passed through, and the predictions (both as a score between 0 and 1 and as a binary outcome) are saved in a csv file. All necessary information to install and use the model is available in the Supplementary Information. However, a MOE license is required to calculate all descriptors needed.

Virtual screening

A total number of 1700 compounds were retrieved from the DrugBank database (www.drugbank.ca, [18]) (as of October 2013) and curated using the same protocol as that described in the Database curation section. These compounds were passed through the model and ranked according to their predicted inhibitor score, which roughly corresponds to their probability of inhibiting BSEP according to the model.

Chemical similarity

Chemical similarity was measured by Tanimoto similarity on RDKit fingerprints [19].

***In vitro* testing**

Materials

[^{14}C]-taurocholic acid and [^{3}H]-taurocholic acid were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Taurocholic acid was obtained from ICN Biomed, Inc. (Irvine, California, USA). Cyclosporin A, pioglitazone, taurocholate, ATP, $Mg(NO_3)_2$ and sodium butyrate were from Sigma (St. Louis, MO, USA). TRIS and Hepes were purchased from Roth (Karlsruhe, Germany), sucrose from neoLab (Heidelberg, Germany), and KNO_3 and KCl from Merck (Darmstadt, Germany). All other compounds were obtained from Glentham Life Sciences Ltd (Wimbledon, London, United Kingdom). LLC-PK1 stable cell lines expressing BSEP and NTCP were generated in the laboratory of B. Stieger. The expression analysis of BSEP was performed by western blot analysis and is available as Supplementary Information. CHO Flip-In cells expressing NTCP were characterized in [20]. The liquid scintillation fluid Filter Count, used in transport studies, was purchased from PerkinElmer (Waltham, MA, USA).

Cell culture

LLC-PK1 cells stably expressing BSEP and NTCP were cultured in DMEM (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS), 2 μg/mL puromycin and

700 µg/mL G418. Chinese hamster ovary (CHO) Flip-In cell lines were cultured in Ham's F-12 medium (Gibco-Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing 100 IU/mL penicillin/streptomycin, 10% fetal calf serum, 1 mM l-glutamine. CHO WT cells were maintained in the presence of 0.1 mg/mL Zeocin (Invitrogen, Waltham, Massachusetts, USA) whereas CHO-NTCP cells were cultured with 0.5 mg/mL hygromycin B (Invitrogen, Waltham, Massachusetts, USA). All cells were cultured at 37°C in an atmosphere containing 5% CO2 with 95% relative humidity.

Taurocholate transport studies in BSEP-expressing Hi5 membrane vesicles

ATP-dependent uptake of [^{3}H]-taurocholate by BSEP was measured using human BSEP-expressing Hi5 membrane vesicles (Solvo, Budapest, Hungary) and the rapid filtration technique as previously described [20]. Vesicles were diluted to a concentration of 2.5 µg/µL with 50 mM sucrose, 100 mM KNO_3, 20 mMHepes/Tris pH 7.4; 10 µL of vesicles was pre-warmed in a 37°C water bath for 30 sec, and uptake was initiated by the addition of 40 µL uptake buffer (50 mM sucrose, 100 mM KNO_3, 12.5 mM $Mg(NO_3)_2$, 10 mMHepes/Tris, pH 7.4) containing radiolabelled taurocholate (3 µCi/mL; final concentration in the assay 2.5 µM). After 10 min of incubation in the 37°C water bath, transport was stopped by adding 3 mL of ice-cold stop solution (50 mM sucrose, 100 mM KCl, 10 mM Tris/HCl, pH 7.4) and immediate filtration through 0.45 µm nitrocellulose acetate filter (Sartorius, Göttingen, Germany) pre-soaked with 1 mM taurocholate solution. The tube was rinsed once with 3 mL of cold stop solution and also filtered. After filter washing with 3 mL of ice-cold stop solution twice, the filter was dissolved in liquid scintillation fluid and radioactivity was measured in a Beckman Counter. For inhibition studies, the compounds were added to the uptake buffer to reach a final concentration of 10 µM. The final DMSO concentration in all samples was 0.8%. As recommended by the manufacturer, cyclosporin A was used as positive control at a final concentration of 20 µM. In addition, pioglitazone was used as positive control at a final concentration of 10 µM. To measure ATP-dependent taurocholate (TC) uptake, 5 mM ATP (final concentration in the assay) was added to the uptake buffer, and uptake rates were calculated as the difference in uptake in the presence and in the absence of ATP.
Hi5-beta galactosidase membrane vesicles (Solvo, Budapest, Hungary) were used as negative control to measure endogenous TC transport of Hi5 membrane vesicles.

IC_{50} determination of bromocriptin in BSEP-expressing Hi5 membrane vesicles

ATP-dependent uptake of [^{3}H]-taurocholate by BSEP was measured in absence and presence of 8 different concentrations of bromocriptin at an incubation time of 5 min as described in the section above. For IC_{50} measurements of bromocriptin, stock solutions were prepared by a 1:2.5 serial dilution in DMSO, resulting in final bromocriptin concentrations ranging from 50 to 0.08 µM. Final DMSO concentration in all samples was 0.8 %. The uptake rates, calculated as the difference in uptake in the presence and in the absence of ATP, were plotted against the logarithmic concentration of bromocriptin in µM. IC_{50} values were calculated by nonlinear regression analysis using GraphPad Software Inc. (San Diego, CA, USA) Prism version 6.00 and the equation

$$y = y_{min} + \frac{(y_{max} - y_{min})}{1 + 10^{(\log IC_{50} - x).a}}$$

where *a* is the Hill Slope.

Taurocholate transport studies in LLC-PK1 NTCP- and BSEP-expressing cells

The transcellular assay was set up following the methodology reported in Kagawa *et al*. [21] with some modifications. Briefly, LLC-PK1 stable cell line expressing BSEP and NTCP were plated on a Transwell® membrane insert (no. 3470, diameter 6.5 mm, pore size 0.4 µm, Corning Costar, Cambridge, MA, USA) at a concentration of 2 x 10^5 cells/membrane. After 24 h, the cells were treated with 5 mM sodium butyrate for further 24 h. To measure taurocholic acid transport activity, cells were washed twice with PBS and moved to DMEM (without FCS or antibiotic). Subsequently, the cells were incubated with pre-warmed basal medium containing 10 µM [^{14}C]-taurocholic acid and pre-warmed apical compartment in the presence or absence of testcompounds. After 1 h incubation under humidified condition at 37°C with 5% CO_2, the apical medium was collected for measurement of the radioactivity using a scintillation counter (Tri-Carb 2100TR, PerkinElmer).

Taurocholate transport studies in CHO NTCP-expressing cells

[^{14}C]-Taurocholic acid uptake and inhibition of NTCP were performed in CHO-WT and CHO Flip-In NTCP cells as described previously [22]. Cells were plated at the density of 1 x 10^5 cells/well onto 24-well plates. Twenty-four hours after seeding, the cells were treated with 5 mM sodium butyrate and cell culture was pursued for further 24 h prior to the experiment.
Before the uptake experiment, CHO cells were rinsed three times with 1 mL of pre-warmed uptake buffer and then pre-incubated in 500 µL of pre-warmed buffer before adding substrate. To measure Na-driven NTCP-mediated transport, two different uptake buffers were used either with sodium (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 20 mM Hepes/Tris pH 7.4) or without (116.4 mM choline chloride, 5.3 mM KCl, 1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 20 mM Hepes/Tris pH 7.4). Taurocholic acid uptake was initiated by the addition of 500 µL pre-warmed uptake buffer containing 2.5 µM taurocholic acid (0.025 µCi/mL [^{14}C]-taurocholic acid). After 5 min of incubation at 37° C, the uptake was stopped by removal of the uptake solution followed by washing 3 times with 1 mL ice-cold uptake buffer. The cells were then lysed in 500 µL of 0.1 mM NaOH for at least 1 h at room temperature. The lysate suspension was used for radioactivity measurement by liquid scintillation counting.
For the inhibition studies, the uptake was examined in the presence of positive control (cyclosporin A) or test compounds at a final concentration 10 µM. DMSO was adjusted to 1% as a control in all experiments.

Statistical analysis

Statistical analyses were performed by using unpaired, parametric t-test using GraphPad Software Inc. (San Diego, CA, USA) Prism version 6.00. Data were normalized to DMSO control, which was set as 100%. T-test was applied to compare each single compound with the DMSO control.

Results

Classification model

As outlined in the methods section, a panel of classifiers was trained on a set of 670 compounds in order to predict if a compound acts as BSEP inhibitor. The performance of the

best model, a Random Forest of 10 trees with integrated feature selection based on a set of 78 1D and 2D descriptors, is reported in Table 2. A charged and a neutralized version of the training set were used and the neutralized version gave the best cross-validation results.

	TN	FN	TP	FP	MCC	ROC area
10-fold CV	408	65	155	42	0.63	0.91
5-fold CV	409	68	152	41	0.62	0.91
Test set	97	18	37	16	0.54	0.87
External set	108	9	30	9	0.69	0.92

Table 2: Confusion matrix, Matthews correlation coefficient and area under the ROC curve for the cross-validation and test sets for the best model.

On the basis of the features selected by the model (number of aromatic atoms (a_aro), number of triple bonds (b_triple), Van der Waals surface area of H bond donors (vsa_don), number of Br atoms (a_nBr), logP (logP(o/w)), Van der Waals surface area of hydrophobic atoms (vsa_hyd), number of C atoms (a_nC), number of rings (rings), molecular weight (weight)), hydrophobicity, the size of the molecule, the aromaticity and the number of hydrogen bond donors seem to play a critical role in BSEP inhibition.

Virtual screening

Given the good performance of the model in internal and external validation, DrugBank was screened for potential BSEP inhibitors. For this, 1700 small molecules from the DrugBank database [18] were passed through the Random Forest and ranked by prediction score. This score takes values between 0 and 1, and the higher the score, the higher the probability of the compound to be a BSEP inhibitor according to the model. 59 compounds actually got the highest possible score, and were further investigated. Among them, 13 compounds were labeled as inhibitors in the training set used to build the model. 10 others were reported in the literature or in DrugBank as inhibitors of BSEP but were not part of the training set. Irinotecan and atorvastatin were reported as non-inhibitorand inhibitor at 50 µM, respectively [10]. Since we used a threshold of 10 µM to define an active in the training set, we count both results as false positives. Tamoxifen and nelfinavir are inconsistently reported with both inhibitor and non-inhibitor activities [8,16,10,13]. This leaves us with 32 compounds that are top ranked and for which we could not find any pharmacological annotation regarding BSEP.

A further analysis of the results of the screening for scores down to 0.8 (still predicted as inhibitors, but with less probability) retrieved 26 compounds with known BSEP annotation: 8 compounds that were present in the training set, 12 true positive (*i.e*. compounds found in literature to be inhibitors), 4 compounds with middle range activities and 2 false positive (*i.e*. compounds found in the literature to be non-inhibitors of BSEP).

On the other hand, 1340 DrugBank small molecules received a score below 0.5, which is the threshold used for binary prediction. This means that the model predicts those compounds as non-inhibitors of BSEP. We studied these compounds and found 314 for which there was a pharmacological annotation available. 147 of them were actually part of the training set of the model (including 2 misclassified compounds), while 161 compounds were reported in the literature as non-inhibitors but were not part of the training set. Another 18 compounds had IC_{50} values over 10 µM but below 50 µM, and as such do not strictly count as errors

since we used 10 µM as a threshold to define the inhibitors in the training set. Finally, six compounds were mispredicted as non-inhibitors while they were actually reported as inhibitors: atazanavir [10,23], telithromycin [8,16], ranolazine [10], trovafloxacin [23], pioglitazone [13,9,16] and diethylstilbestrol [10]. This results in 161 true negatives and 6 false negatives, which corresponds to a negative predictive value of 96% (the negative predictive value is obtained by dividing the number of true negatives by the total number of predicted negatives). To compare with, the negative predictive value obtained for the 10-fold cross-validation was 86%, 84% for the randomly selected test set and 92% for the external set. The positive predictive value for the top scoring compounds analyzed here is 0.73 (22 true positives and 8 false positives). This value is lower than the one obtained for the external set (0.77), the 10-fold cross-validation (0.79) but higher than the one of the randomly selected test set (0.70).

***In vitro* testing**

Among the 32 compounds which received the highest score in the model and had no reported BSEP interaction in the literature, we eliminated one compound, halobetasol, which has topical application. Fourteen compounds that were not reported to undergo liver metabolism or elimination were removed. To ensure that compounds do not have a Tanimoto similarity > 0.75 to any other compound within the set or in the training set, we removed toremifene, which had a similarity of 0.94 with one compound of the training set. This led to a short list of 16 compounds, whereby 7 of them could finally be purchased.

To directly assess BSEP inhibition, we used a membrane vesicle assay as recommended by the International Transporters Consortium [24]. This kind of assay has the advantage of showing the direct impact of the test compounds on BSEP-mediated taurocholate (TC) transport. It is devoid of metabolism or permeability uptake problems.

The obtained results at a single concentration of test compounds are reported in Figure 1. In addition to the positive controls cyclosporin A and pioglitazone, which indeed decreased TC uptake to 15 ± 3% and 8 ± 3% respectively, bromocriptin reduced BSEP-mediated TC uptake significantly to 45 ± 10%. In contrast, raloxifen and vecuronium increased BSEP-mediated TC transport significantly to 125 ± 7% and 114 ± 7%, respectively, as compared to the DMSO control. All other compounds did not affect BSEP function. Vesicles which did not express BSEP were used as negative control to exclude TC transport by endogenously expressed transporters in Hi5 cells. Indeed, these vesicles showed only 3 ± 1% of TC transport compared to vesicles expressing BSEP.

The inhibitory activity of bromocriptin was studied in more detail by an IC_{50} value determination. As shown in Figure 2, an IC_{50} value of 1.2 ± 0.1 µM was evaluated.

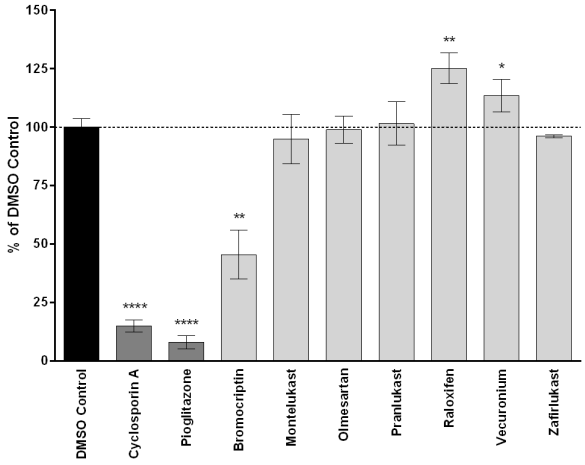

Figure 1: Effect of different compounds on BSEP-mediated TC transport. ATP-dependent uptake of 2.5 µM TC in BSEP-Hi5 membrane vesicles was measured in absence (black bar) and presence of the test compounds (light grey) or known BSEP inhibitors (dark grey) as described in the materials and methods section. TC uptake data are given as the mean percentage of DMSO control ± SD of 3 independent experiments. Each experiment was performed in technical triplicates. Compounds showing significant different TC uptake as compared to DMSO control are marked with asterisks (*, $p<0.05$; **, $p<0.01$; ****$p<0.0001$).

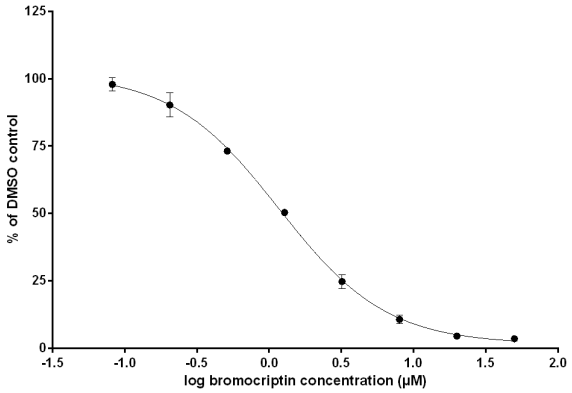

Figure 2: Effect of bromocriptin on BSEP-mediated TC transport. ATP-dependent uptake of 2.5 µM TC in BSEP-Hi5 membrane vesicles was measured in absence and presence of eight increasing concentrations of bromocriptin as described in the Materials and Methods section. Data are given as the mean percentage of TC uptake compared to the DMSO control ± SD of 3 independent experiments. Each experiment was performed in technical triplicates.

In an attempt to evaluate BSEP inhibition in a whole-cell system, we also tested the seven compounds in a transcellular flux assay. For this, we used LLC-PK1 cells expressing NTCP at the basolateral and BSEP at the apical membrane (see Supplementary Information, Figure SI-3). NTCP mediates the uptake of taurocholate from the basal compartment, while BSEP exports taurocholate into the apical medium. The final amount of transported substrate is measured from the apical compartment. The inhibition results at a single concentration (10 µM) of test compounds are shown in Figure 3. The positive controls, cyclosporin A and pioglitazone, showed lower taurocholate transport (42 ± 11% and 55 ±

18%, respectively). Bromocriptin was found again to cause a decreased transport of taurocholic acid (69 ± 5%). In addition, montelukast showed an inhibitory effect (57 ± 18%).

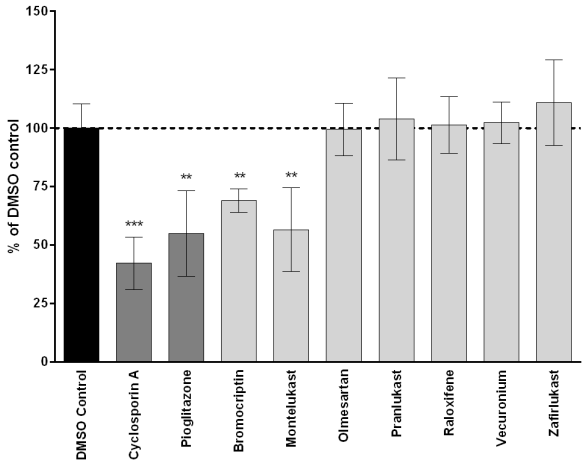

Figure 3: BSEP inhibition study in a transcellular flux assay. Effect of known BSEPinhibitors (cyclosporin A and pioglitazone, dark grey bars) and test compounds (light grey) was studied using LLC-PK1 cells stably expressing BSEP and NTCP in a Transwell® system as described in the Materials and Methods section. Data show the amount of taurocholate in the apical compartment medium as a percentage of the DMSO control (absence of compound). Data are given as the mean percentage of taurocholate uptake compared to the DMSO control (black bar) ± SD of at least 3 independent experiments. Compounds showing significant different TC amount in the apical compartment as compared to DMSO control are marked with asterisks (**, $p<0.01$; ***, $p<0.001$).

As previously described, the transcellular transport of taurocholate (TC) through a cell monolayer is mediated by the sodium-dependent TC uptake transporter NTCP at the basolateral side and the ATP-dependent TC efflux via BSEP at the apical side. As montelukast showed no inhibitory effect on BSEP in the vesicle assay, but showed inhibition of the transcellular flux of TC, we investigated the effect of the known NTCP inhibitor cyclosporin A as well as bromocriptin and montelukast on NTCP-mediated TC transport. Cyclosporin A shows a strong inhibition at 10 µM (13 ± 1**%**). Interestingly, montelukast also inhibits taurocholic acid uptake by NTCP (46 ± 7**%**), while bromocriptin shows no significant effect compared to the DMSO control (see Supplementary Information, Figure SI-4).

Discussion

After pharmacokinetics problems and lack of efficacy, the toxicity of compounds is a major cause of failures in late stages of drug development [25]. In particular, DILI is the most frequent reason for withdrawal of approved drugs [26]. Early identification of such problematic compounds must be a priority for obvious economic and social reasons.

BSEP inhibition has been correlated with drug-induced liver injury (DILI) [13]. As bile salt transporter localized at the canalicular membrane of hepatocytes, BSEP is the major

constituent to generate bile flow, mediating the transport of monovalent bile salts into bile [27]. Any impairment of BSEP function results in bile acid accumulation and consequently liver injury due to the bile acid cytotoxicity [28,29,30,31,32,33,34,35]. Dawson and colleagues [13] studied the relationship between drug-induced hepatotoxicity and the potency of BSEP inhibition for 85 pharmaceuticals. They found out that all the 17 drugs in their dataset having an IC_{50} for human BSEP inhibition below 100 µM caused DILI. They also found that BSEP inhibitors tend to have high molecular weight and lipophilicity. Similar correlation was found by Pedersen and colleagues [10], where more than half of the BSEP inhibitors in their study were strong DILI inducers.

To the best of our knowledge, three ligand-based models to predict BSEP inhibition have been reported so far [8,9,10]. Briefly, Hirano and colleagues fitted a QSAR model to predict the percentage of inhibition by linear regression on 37 compounds [8]. They report a R^2 of 0.95 but did not perform a cross-validation to evaluate the predictivity of their model. More recently, Warner and colleagues used a larger dataset (437 compounds) to build a binary classification model to predict BSEP inhibition. The best external validation performance was obtained with support vector machine built on in-house descriptors (accuracy: 0.87, sensitivity: 0.9, specificity: 0.84) [9]. Finally, Pedersen and colleagues performed partial least-square discriminant analysis to classify BSEP strong inhibitors, weak inhibitors and non-inhibitors [10]. The best model was obtained when using only the two classes "strong inhibitors" versus "non-inhibitors". In that case, the training set contained 142 compounds and the test set 74 compounds (accuracy: 0.89, sensitivity: 0.76, specificity: 0.94).

Our own models were built on a significantly larger training set (670 compounds) and validated by cross-validation and external sets. The external validation gives an accuracy of 0.88, sensitivity of 0.77 and a specificity of 0.92 (Table 2). This is equivalent to the values reported in [9,10]. The high area under the ROC curve (AUC, around 0.9 for all validation schemes) shows a great capacity for our model at ranking compounds by their probability of being inhibitors of BSEP. The AUC is a good metrics for virtual screening applications and present the advantage of being independent from class distributions [11,36].

The model is extremely fast (the longest step being the computation of the molecular descriptors) and can be used on a large number of compounds in a timely manner. As a reference, we needed around 2 min in a regular desktop computer to predict the 1700 DrugBank compounds. The implementation into a KNIME workflow allows pipelining the whole process, so that there is no need to load the whole library of compounds in the memory. Instead, the molecules to predict are treated one by one, which allows to process libraries of any size.

Such *in silico* models, because of their ability to treat millions of compounds in a timely manner, could in theory be used to pre-filter large libraries of compounds. There are, however, a certain number of pitfalls that should be noted. First, models, even the best ones, make mistakes. That means that one can filter out compounds that actually would not have caused any BSEP inhibition. Or, on the contrary, compounds with bad BSEP profiles might sneak in the filtered database. In this sense, *in silico* models won't replace *in vitro* testing, they rather aid in prioritizing compounds for being tested or not. Second, ligand-based models depend on their training set. The larger, more diverse the training set is, the more likely it is that the model will perform well on a set of new compounds. It is common practice in the industry to regularly update and retrain models. Indeed, as time passes, the chemical focus of a company may switch and lead to an increase of errors of an initially well-performing model. So retraining the model using an updated chemical space helps stabilizing

the error rate of the models. Also, the quality of the pharmacological data behind the training set is crucial. If many different assays were used to generate this data, then a proper handling and assay-per-assay evaluation should be performed before even building a model [37]. Otherwise, noisy and even disagreeing data may be taken as training set and lead to poor models. In this study, we were in the ideal case where all data used to build the model came from a single assay, performed within the same company.

In the absence of a crystal structure for BSEP, we lack knowledge on the interaction pattern by which drugs inhibit the transporter at the molecular level. The embedded feature selection reveals a set of descriptors related to hydrophobicity, size of the molecules, as well as number of H bond donors as critical for assigning inhibitor/non-inhibitor class labels. The latter seems contradictory to the results of Warner *et al.* [9], who find similar tendencies regarding molecular weight and lipophilicity for the inhibitor set, but an opposite relationship for H bond donors: "*… molecules with an increasing number of donors were at reduced risk of BSEP inhibition ($p < 0.0001$)*". This apparent discrepancy in our respective findings could be the result of the neutralization step applied to our compound structures before computing descriptors. Indeed, BSEP transports negatively charged compounds, and neutralization of such acids leads to strong H-bond donor features in the structure. There is no information in [9] regarding the pre-processing of chemical structures the authors may have applied. In this study, we tried both charged and uncharged versions of the training set to build the models, and it happened that the neutralized version gave higher cross-validation results.

The model was used to prospectively screen DrugBank and proved a good recognition of already known inhibitors and non-inhibitors. In a later step, seven diverse compounds with unknown BSEP activity at the time of the screening were bought and tested *in vitro*. This led us to identify one new inhibitor of BSEP, while the six other compounds were false positives. These six compounds were chemically dissimilar from the training set (Table 3), which could explain the errors of the model. When taking together all the results from the DrugBank screen (including the top ranking compounds for which we did find BSEP inhibition in the literature), we obtain the following figures: 23 true positives (compounds that were identified either in the literature or in our assay as true positives), and 14 false positives (when taking a strict threshold at 10 µM to define an active). This corresponds to a moderate positive predictive value of 0.62. While this can seem a high false alarm rate, one has to remember that it is the first time that an *in silico* model for BSEP has been actually used in such a prospective way. The usefulness of the model is actually to quickly sort out "safe" compounds (with high success, since we could find only 6 false negatives in the DrugBank screen) from potentially "troublesome" compounds. If one thinks of a safety assessment pre-screening, then it is actually quite desirable to have such a conservative model (*i.e*. a model with many false positives).

	Similarity to closest neighbor	Average similarity
Bromocriptin	0.75	0.44
Montelukast	0.51	0.35
Olmesartan	0.61	0.40
Pranlukast	0.60	0.36
Raloxifen	0.52	0.37
Vecuronium	0.56	0.41
Zafirlukast	0.56	0.40

Table 3: Tanimoto similarity between the seven tested compounds and the training set.

The vesicle assay identified bromocriptin as inhibitor of BSEP function. To the best of our knowledge, the effect of bromocriptin on BSEP function has not been investigated yet. Bromocriptin has been approved for the treatment of hyperprolactinemia syndromes, prolactinomas, acromegaly and Parkinson's disease in 1977. In 2009, bromocriptin mesylate was additionally approved for the treatment of type 2 diabetes [38]. Although we showed clear inhibitory effect on BSEP function, this drug is not known to cause cholestasis. According to the LiverTox database [39], bromocriptin has been reported to trigger an asymptomatic elevation of serum aminotransferase levels. Only rare instances of acute liver injury have been reported and no case reports were published. Bromocriptin is therefore considered as a safe drug regarding liver injury. The absence of DILI may be due to extensive metabolism of bromocriptin, essentially by the cytochrome CYP3A subfamily [40,41], which most likely leads to low intracellular concentration of bromocriptine in hepatocytes. In addition, unbound plasma concentrations of bromocriptin are about 60 times lower (15 nM, [42]) than the IC_{50} of bromocriptin in the vesicle assay (1.2 µM).

Isolated membrane vesicles as well as the cell-based transcellular flux assay are common methods to study efflux transporters. We demonstrated inhibitory effects of cyclosporin A, pioglitazone and bromocriptin on BSEP function in both systems, even if this effect was more pronounced in isolated membrane vesicles. The observed difference in inhibition activity could be due to the fact that, in the inside-out vesicle assay, the test compound is directly applied to the interaction site of BSEP. On the other hand, in the transcellular assay, the exact amount of test compounds entering the cell and being available for interaction with BSEP is not known. Therefore, potential differences in concentrations at the active site may arise, leading to different inhibitory activities. Most probably, this variability between the two assays is not caused by the known difference in cholesterol content of insect and mammalian cell membranes. Indeed, although cholesterol does affect the transport kinetics of taurocholate by BSEP and the inhibitory activity of cyclosporine A, it does so by only affecting Vmax but not Km values [43,44]. The only controversial result found in membrane vesicle and cell-base inhibition studies was the effect of montelukast. While this compound had no effect on BSEP function in isolated membrane vesicles, an inhibitory activity comparable to the effect of bromocriptin was shown in the Transwell® assay. Like for bromocriptin, no published data are available investigating the effect of montelukast on BSEP function. But this compound has been reported to induce liver injury in pediatric and adult patients, which cannot be related to bile acid accumulation and hepatic transporter inhibition to date [45,46]. Our study here clearly demonstrates no cis-inhibitory effect of this compound on BSEP in the vesicle-based assay. If added apically in the cell based transflux assay, an inhibitory effect is indeed observed in the basolateral to apical flux of taurocholate. To evaluate whether this effect could be due to the inhibition of the primary step in the transflux assay (the NTCP-mediated uptake of TC), the effect of montelukast was studied in NTCP-expressing CHO cells. Montelukast showed significant inhibition of NTCP-mediated taurocholate uptake, which may explain the effects seen in the Transwell® assay. However, further evaluation is needed to verify this hypothesis.

While we were performing our screening experiments, Morgan and coworkers published a new study on hepatobiliary transport inhibition and its link with liver injury [47]. As part of their study, they measured BSEP inhibition of 600 drugs in a vesicle assay. Among the

tested compounds, olmesartan was reported with an IC_{50} of 4.73 µM, pranlukast with an IC_{50} of 2.97 µM, vecuronium with an IC_{50} over 100 µM and zafirlukast with an IC_{50} of 11.1 µM. These results, apart for vecuronium, are in contradiction with the absence of inhibition at 10 µM found in both our vesicle and transcellular transport assays. In our opinion, the most likely explanation for the discrepancy in the results is of technical nature. In our assay, the uptake starts by mixing vesicles with the uptake solution containing the substrate, the inhibitor and ATP. This corresponds to true zero-trans conditions. In contrast, as described in details by van Staden and co-workers [48], Morgan *et al*. start the uptake by adding ATP to vesicles pre-incubated with both substrate and inhibitors. This pre-incubation leads most likely to a deviation from a true zero-trans condition, which is needed for this type of transport experiment.

All in all, these results point towards the importance of choosing an appropriate assay system for validating results of *in silico* screens. Ideally, the same method as was used in the training set of the model should be employed. However, this is not always possible, especially if diverse data sources are merged together. In general, a model as simple as possible (a single protein overexpressed, an isolated vesicle, etc.) is recommended to limit the noise brought by possible other interaction partners. More complex assay systems are of great help to understand mechanisms and mimic as much as possible *in vivo* effects.

Conclusion

In the present work, an *in silico* model based on a large set of compounds has been developed to identify potential BSEP inhibitors that could induce cholestasis. Best performance was obtained by RandomForest with embedded feature selection algorithm as classifier. Subsequent *in silico* screening of DrugBank followed by experimental testing of top ranked hits allowed the discovery of bromocriptin as previously unknown BSEP inhibitor.

In conclusion, this modeling approach allows to quickly flagging compounds with potential inhibition activity at BSEP that could in theory show drug-induced cholestasis. The implementation of this model in a KNIME workflow for easy use is a first step for creating a tool for *in silico* transporter profiling including also the already established models for P-glycoprotein [49], the breast cancer protein [37], as well as ABCC2 [14].

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Supporting Information

The authors provide as Supplementary Information:

- the detailed list of the names of the MOE descriptors computed in this study

- an explanation of the software requirement and installation process to run the KNIME workflow
- the characterization of the LLC-PK1 cells
- the Transwell® assay set-up
- the results for NTCP inhibition in NTCP-expressing CHO cells for cyclosporin A, pioglitazone, bromocriptin and montelukast
- the KNIME workflow to run BSEP inhibition predictions using the model described in this study (.zip archive)

This material is available free of charge via the Internet at http://pubs.acs.org.

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